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Raf-Induced Vascular Endothelial Growth Factor Augments Kaposi's Sarcoma-Associated Herpesvirus Infection

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Recombinant green fluorescent protein encoding Kaposi's sarcoma-associated herpesvirus (rKSHV.152) infection of β-estradiol stimulated human foreskin fibroblasts (HFF) or HFF/ΔB-Raf$_{[FF]}$:ER (expressing a weaker form of B-Raf) could be enhanced to levels comparable to that of HFF/ΔB-Raf$_{[DD]}$:ER cells by pretreating cells with soluble vascular endothelial growth factor (VEGF). Conversely, VEGF expression and infection efficiency typically observed in β-estradiol stimulated HFF/ΔB-Raf$_{[DD]}$:ER cells could be lowered significantly by treating with VEGF small interfering RNA. In addition, we observed enhancement of the KSHV infection in HFF cells transfected with human VEGF$_{121}$. These results confirm the ability of Raf-induced VEGF to augment KSHV infection of cells.

Kaposi's sarcoma-associated herpesvirus (KSHV), otherwise known as human herpesvirus 8 (HHV-8), is the most recently characterized of the human herpesviruses. KSHV is a member of the γ-2 herpesvirus family (genus *Rhadinovirus*) and was first isolated in 1994 from Kaposi's sarcoma (KS) lesion material in persons suffering from AIDS (10, 50). KSHV is also a lymphoproliferative agent that has been etiologically linked to two types of malignant lymphomas occurring in AIDS patients: primary effusion lymphoma and multicentric Castleman's disease (9, 53).

KS is a neoplasm of vascular origin arising as multiple independent lesions that, over time, can progress into a nodular tumor localizing in the skin and visceral organs, including the gastrointestinal tract and lungs (19). KSHV infects a variety of target cells in vitro, including fibroblasts and endothelial, epithelial, and human B cells (8, 22, 39, 43, 49). In a recently published study, enhanced KSHV infection of cells that expressed different Raf oncoproteins was demonstrated (1). We analyzed the effect of the three related Raf genes, A-Raf, B-Raf and Raf-1 (12, 38), with ΔRaf:ER fusion proteins that become activated upon β-estradiol (EST) treatment (31). The rank order of enhanced KSHV infection observed in human foreskin fibroblast (HFF) cells was ΔB-Raf:ER > ΔRaf-1:ER > ΔA-Raf:ER. In addition, we also found that Raf oncoproteins induce vascular endothelial growth factor (VEGF) expression in cells and that VEGF promotes virus entry into cells. Hence, we analyzed the physiological relevance of the Raf-induced VEGF expression on KSHV infection of target cells.

In this study we used HFF, HFF/pBabePuro3, HFF/ΔB-Raf$_{[FF]}$:ER, and HFF/ΔB-Raf$_{[DD]}$:ER cells. HFF is a primary cell culture, HFF/pBabePuro3 is HFF transfected with empty vector, HFF/ΔB-Raf$_{[DD]}$:ER is HFF expressing wild-type B-Raf, and HFF/ΔB-Raf$_{[FF]}$:ER is HFF expressing B-Raf with a mutation at amino acid position 492 (DD to FF), which results in decreased levels of B-Raf activity. HFFΔB-Raf$_{[DD]}$:ER cells stimulated with EST express significantly higher levels of Raf activity when compared to unstimulated and EST stimulated HFF, HFF/pBabePuro3, and HFF/ΔB-Raf$_{[FF]}$:ER cells and unstimulated HFFΔB-Raf$_{[DD]}$:ER cells (1). KSHV infection of EST-stimulated HFFΔB-Raf$_{[DD]}$:ER cells was significantly higher than that observed with EST-stimulated HFF, HFF/pBabePuro3, and HFF/ΔB-Raf$_{[FF]}$:ER cells (1). We chose these cells due to the differences in the permissiveness to KSHV infection, which is directly proportional to the strength of the Raf activity.

Soluble VEGF enhances rKSHV.152 infection. A major finding in our previous study was a positive correlation observed in cells between the expression of VEGF and Raf activity. In this study, we wanted to examine whether soluble VEGF could enhance KSHV infection of cells. VEGF is an angiogenic factor expressed in KS lesions and known to play a key role in KS pathogenesis (19, 20, 42).

Recombinant green fluorescent protein (GFP) encoding KSHV (rKSHV.152) was used to monitor virus infection (56). rKSHV.152 infections were routinely performed at a multiplicity of infection (73 IU) of 0.1 per cell (44). VEGF significantly enhanced rKSHV.152 infection of unstimulated HFF and HFF/ΔB-Raf$_{[FF]}$:ER cells (Fig. 1A). Similar results were observed in unstimulated HFF/pBabePuro3 and HFF/ΔB-Raf$_{[DD]}$:ER cells and EST-stimulated HFF, HFF/pBabePuro3, and HFF/ΔB-Raf$_{[FF]}$:ER cells (data not shown). A concentration-dependent enhancement of rKSHV.152 infection of cells was observed after treatment of HFF cells with VEGF. A maximal enhancement (4.6 ± 0.3 fold in VEGF-treated versus 1 fold in untreated cells) was observed when HFF cells were treated with 1 μg of VEGF/ml (Fig. 1A). Interestingly, VEGF enhanced rKSHV.152 infection of EST-stimulated HFF/ΔB-Raf$_{[DD]}$:ER cells only to a modest extent over the untreated

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cells (Fig. 1A). EGF did not significantly alter the rKSHV.152 infection of all the cell types tested in this study (Fig. 1A). The above infections were also monitored and confirmed by staining for ORF73 expression by immunoperoxidase assay (Fig. 1B to E) and reverse transcription-PCR (RT-PCR) (Fig. 1F).

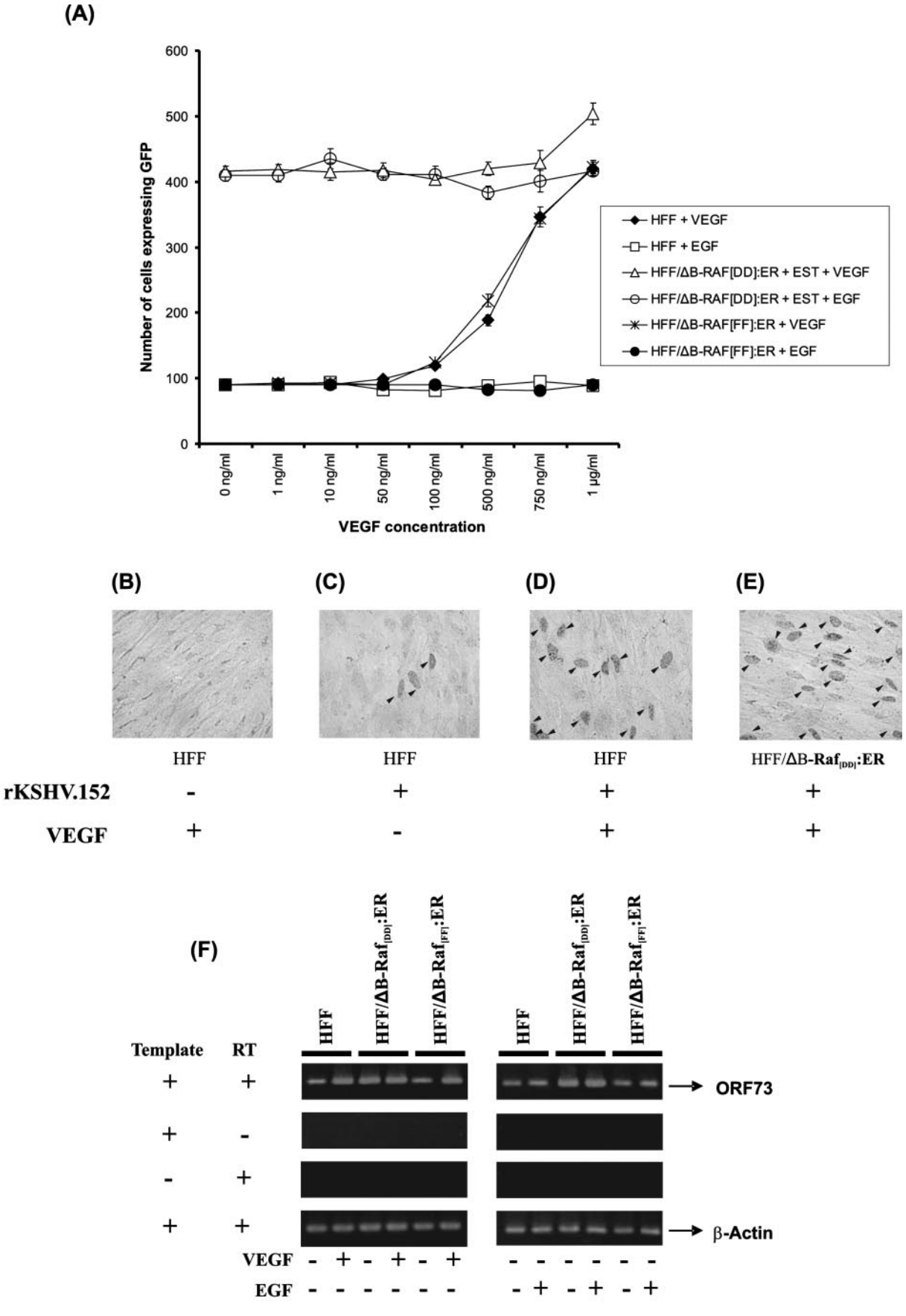
Radiolabeled rKSHV.152 bound readily and to comparable extents in unstimulated (data not shown) and EST-stimulated HFF and HFF/ΔB-Raf$_{[FF]}$:ER cells that were pretreated with VEGF (Fig. 1G), irrespective of the infection pattern (Fig. 1A). rKSHV.152 bound to both untreated and VEGF-treated (1 μg/ml) cells to a comparable extent. Heparin (H) at a concentration of 10 μg/ml significantly inhibited (by about 90%) the ability of rKSHV.152 to bind the unstimulated (data not shown) and EST-stimulated HFF and HFF/ΔB-Raf$_{[FF]}$:ER cells that were pretreated with VEGF (Fig. 1H). In contrast, 10 μg/ml of chondroitin sulfate A (CSA) did not have any significant effect on binding of rKSHV.152 to EST-stimulated target cells that were pretreated with VEGF (Fig. 1G). These results demonstrate that VEGF did not enhance the ability of virus to bind cells. We concluded that VEGF enhances virus infection at a postattachment stage of entry.

HFF/ΔB-Raf$_{[DD]}$:ER cells express higher levels of VEGF. Our results demonstrated the ability of VEGF to enhance KSHV infection of HFF and HFF/ΔB-Raf$_{[FF]}$:ER cells (Fig. 1A). Based on these results, we hypothesized that EST-stimulated HFF/ΔB-Raf$_{[DD]}$:ER cells expressed higher levels of VEGF than unstimulated or EST-stimulated HFF and HFF/ΔB-Raf$_{[FF]}$:ER cells. Hence, we quantitated VEGF expression in these cells by performing an enzyme-linked immunosorbent assay (ELISA). EST-stimulated HFF/ΔB-Raf$_{[DD]}$:ER cells produced significantly higher concentrations of VEGF than EST-stimulated HFF and HFF/ΔB-Raf$_{[FF]}$:ER cells (Fig. 1H). The VEGF concentrations in EST-stimulated HFF and HFF/ΔB-Raf$_{[FF]}$:ER cells never exceeded 5 pg/ml. VEGF expression in unstimulated HFF, HFF/ΔB-Raf$_{[FF]}$:ER, and HFF/ΔB-Raf$_{[DD]}$:ER cells as well as in stimulated HFF and HFF/ΔB-Raf$_{[FF]}$:ER cells was comparable to that observed in the EST-stimulated HFF cells (Fig. 1H).

In addition, we analyzed the expression of VEGF isoforms in HFF cells. There are at least five different forms of VEGF (VEGF$_{121}$, VEGF$_{145}$, VEGF$_{165}$, VEGF$_{189}$, and VEGF$_{206}$) that are expressed by cells based on the number of amino acids comprising the protein product after differential splicing (21). HFF cells express VEGF$_{121}$, VEGF$_{145}$, VEGF$_{165}$, and VEGF$_{189}$ (Fig. 1I). HFF does not express the VEGF$_{206}$ isoform, which appears to have a restricted expression only in embryonic tissue (45).

A VEGFR tyrosine kinase inhibitor inhibits KSHV infection of HFF/ΔB-Raf$_{[DD]}$:ER cells. Tyrosine kinases transduce extracellular signals to elicit intracellular responses. VEGF interacts with the target cells and mediates signaling via binding VEGF receptor (VEGFR) tyrosine kinases. Hence, we tested the effect of a VEGFR tyrosine kinase inhibitor on rKSHV.152 infection of cells. The VEGFR tyrosine kinase inhibitor used in this study was a small molecule inhibitor of tyrosine kinase activity with the chemical formula 4-[(4′-chloro-2′-fluoro) phenylamino]-6,7-dimethoxyquinazoline (Calbiochem, San Diego, Calif.). Dimethoxyquinazolines disrupt receptor signaling through interactions with ATP binding sites and have been shown to inhibit nucleoside transport and uptake (32). This

FIG. 1. (A) VEGF enhances rKSHV.152 infection of HFF and HFF/ΔB-Raf$_{[FF]}$:ER cells. Target cells grown for 24 h in either the absence or presence of 1 μM EST were treated with either Dulbecco modified Eagle medium (DMEM) alone or DMEM containing different concentrations of VEGF and EGF for 1 h at 37°C. These cells were then infected with rKSHV.152 in either the absence or presence of VEGF and EGF, respectively, for 2 h at 37°C. Infection was monitored at the end of 3 days postinfection as per standard procedures. We observed 90 HFF cells expressing GFP that were untreated with VEGF. Data are presented as the number of GFP-expressing cells per well, which directly indicates rKSHV.152 infection. (B to E) Monitoring rKSHV.152 infection by immunoperoxidase assays. After monitoring the infection by counting the number of cells expressing GFP, the cells were fixed in ice-cold acetone and incubated with a monoclonal antibody against HHV-8 ORF73 protein, biotinylated anti-mouse antibodies, and substrate as described previously (3). Representative illustrations of uninfected and infected cells are shown. Arrowheads indicate nuclei of cells expressing the ORF73 protein (magnification, ×200). (F) rKSHV.152 infection of target cells was monitored by RT-PCR to detect ORF73 expression. Briefly, EST-stimulated cells were either treated with DMEM or DMEM containing 1 μg of VEGF or EGF/ml for 1 h prior to rKSHV.152 infection of cells. After 48 h, total RNA was isolated from the infected cells with a Nucleospin RNA II kit (Clontech, Palo Alto, Calif.) as per the manufacturer's recommendations. Extracted RNA was examined for the presence of viral RNA transcripts by RT-PCR (1). A 2-μl sample of cDNA was subjected to PCR analysis with specific primers to determine the expression of HHV-8 ORF73 and the human β-actin gene. PCR-amplified products were subjected to electrophoresis through a 1.2% agarose gel. The product sizes of ORF73 and β-actin were 307 and 838 bp, respectively. The DNA signals from RT-PCR were linear with respect to mRNA concentration for the number of cycles used in the amplification. The bands were scanned, and the band intensities were assessed with the ImageQuaNT software program (Molecular Dynamics). (G) VEGF does not enhance binding of KSHV to target cells. Purified [^{3}H]-thymidine labeled rKSHV.152 (2830 cpm) was incubated with DMEM alone or DMEM containing 10 μg of H or CSA/ml for 1 h at 37°C before being added to either untreated cells or cells treated with VEGF (1 μg/ml) that were EST stimulated. After incubation for 1 h at + 4°C with the virus, cells were washed, lysed with 1% sodium dodecyl sulfate and Triton X-100, radioactivity precipitated with trichloroacetic acidand counted as before (3). Approximately 23% of the input KSHV radioactivity became associated with the cells. (H) EST-stimulated HFF/ΔB-Raf$_{[DD]}$:ER cells express higher concentrations of VEGF. An ELISA was performed to quantify levels of VEGF present in the culture supernatant of EST-stimulated cells. Briefly, when the cells were 70 to 80% confluent (10^6 cells/well), the cells were washed twice in DMEM and further incubated in phenol red-free DMEM supplemented with 5% fetal bovine serum at 37°C. After 24 h of incubation, supernatants were collected in 1.5-ml vials, and spun at 1,000 rpm for 10 min at 4°C to remove the particulates. The resulting supernatant (200 μl) was used to test VEGF expression by ELISA as per the manufacturer's recommendations. (I) HFF cells express VEGF$_{121}$, VEGF$_{145}$, VEGF$_{165}$, and VEGF$_{189}$. Total RNA was extracted from HFF cells, and RT-PCR was performed as described for panel F. VEGF primers used were as follows: sense (5′-ATGAACTTTCTGCTGTCTTGG-3′) and antisense (5′-TCACCGCCTCGGCTTGTCA C-3′) (54). The primers are designed to identify all the five forms of VEGF, as they span all of the exons. PCR products were analyzed on a 2.0% agarose gel. The expected product size of VEGF$_{206}$ is 699 bp. (J) VEGFR inhibitor inhibits rKSHV.152 infection of cells. EST-stimulated cells were either untreated or treated with 50 nM VEGFR inhibitor or with dimethyl sulfoxide (vehicle for the VEGFR inhibitor) for 1 h at 37°C. These cells were infected with rKSHV.152 in either the absence or presence of VEGFR inhibitor and monitored for infection; the data are presented as in legend for panel A. Data presented in panels A, G, H, and I represent the averages ± standard deviation (SD) of three experiments. Average values on the columns with different superscripts are statistically significant ($P < 0.05$) by least-significant difference (LSD).


(A)
Number of cells expressing GFP
600
500
400
300
200
100
0
0 ng/ml
1 ng/ml
10 ng/ml
50 ng/ml
100 ng/ml
500 ng/ml
750 ng/ml
1 μg/ml
VEGF concentration
HFF + VEGF
HFF + EGF
HFF/ΔB-RAF[DD]:ER + EST + VEGF
HFF/ΔB-RAF[DD]:ER + EST + EGF
HFF/ΔB-RAF[FF]:ER + VEGF
HFF/ΔB-RAF[FF]:ER + EGF
(B)
(C)
(D)
(E)
HFF
HFF
HFF
HFF/ΔB-Raf[DD]:ER
rKSHV.152 - + + +
VEGF + - + +
(F)
HFF
HFF/ΔB-Raf[DD]:ER
HFF/ΔB-Raf[FF]:ER
Template RT
ORF73
β-Actin
VEGF - + - + - + - - - - - -
EGF - - - - - - - + - + - +

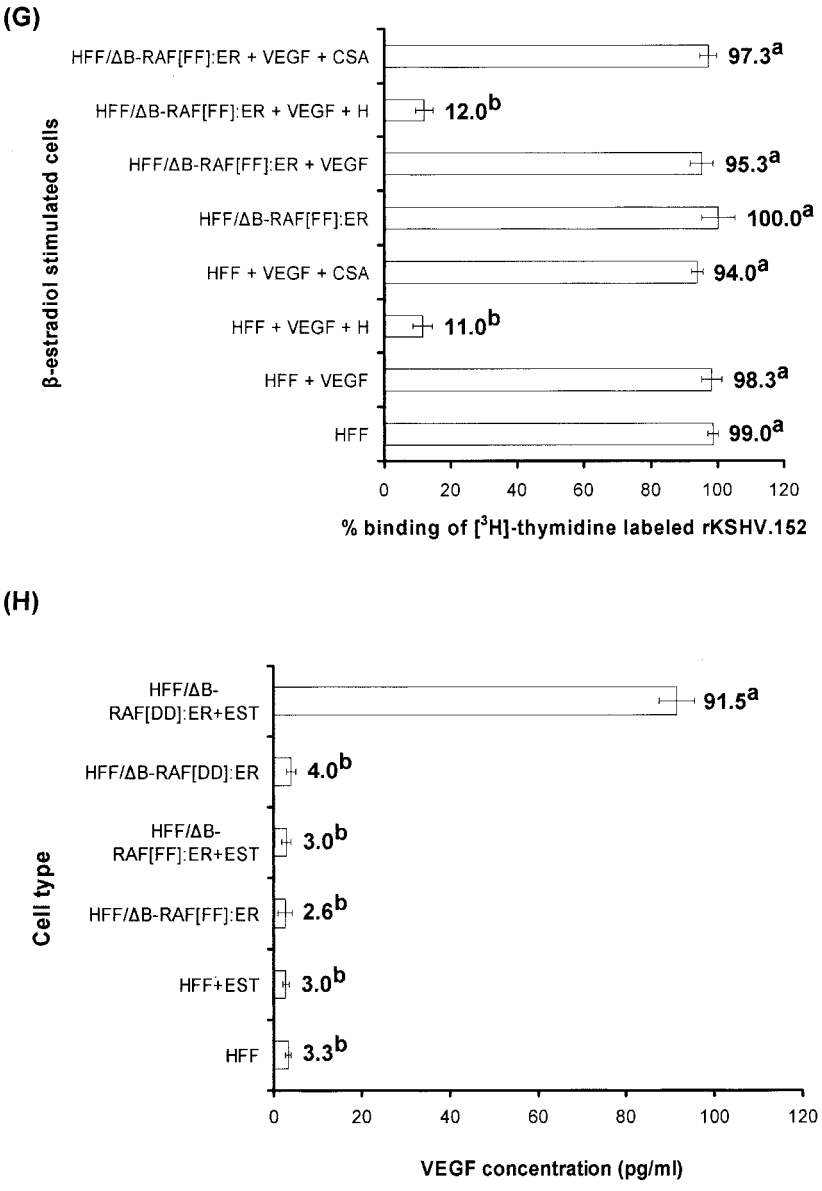


FIG. 1—*Continued.*

inhibitor is both potent and selective for VEGFR1/Flt (for Fms-like tyrosine kinase) and VEGFR2/KDR tyrosine kinase activity compared to the activity of other receptors (30). In our experiments, we used the VEGFR tyrosine kinase inhibitor at a nontoxic concentration (as tested by the CytoTox 96 Non-Radioactive cytoxicity assay; Promega, Madison, Wis.) of 50 nM. Similar concentrations have been used in earlier studies (27, 30). The VEGFR tyrosine kinase inhibitor at 50 nM significantly lowered rKSHV.152 infection of EST-stimulated HFF/ΔB-$Raf_{[DD]}$:ER cells (Fig. 1J). The VEGFR tyrosine kinase inhibitor did not alter infection of either EST-stimulated HFF, HFF/ΔB-$Raf_{[FF]}$:ER (Fig. 1J), and HFF/pBabePuro3 cells or unstimulated HFF, HFF/pBabePuro3, HFF/ΔB-$Raf_{[DD]}$:ER, and HFF/ΔB-$Raf_{[FF]}$:ER cells (data not shown). No significant inhibition in rKSHV.152 infection of cells was observed when dimethyl sulfoxide (a vehicle for VEGFR tyrosine kinase inhibitor) was used at a similar volume (Fig. 1J). We also tested the effect of VEGFR tyrosine kinase inhibitor on herpes simplex virus type 2 (HSV-2) infection of cells as per earlier protocols (1). Interestingly, in contrast to KSHV infection, there was no significant drop in HSV-2 infection (at a multiplicity of infection of 0.1) of EST-stimulated HFF/ΔB-$Raf_{[DD]}$:ER cells. A 50% tissue culture infective dose of approximately $10^{6.5}$ of HSV-2 was produced in EST-stimulated HFF and HFF/ΔB-$Raf_{[DD]}$:ER cells that were either untreated or treated with the inhibitor. This data also demonstrated the specificity of the effect of the VEGFR tyrosine kinase inhibitor on KSHV infection. These results indicate that signaling via VEGFR is not an absolute necessity for infection and that signaling via VEGFR plays a role in augmenting KSHV infection of target cells.

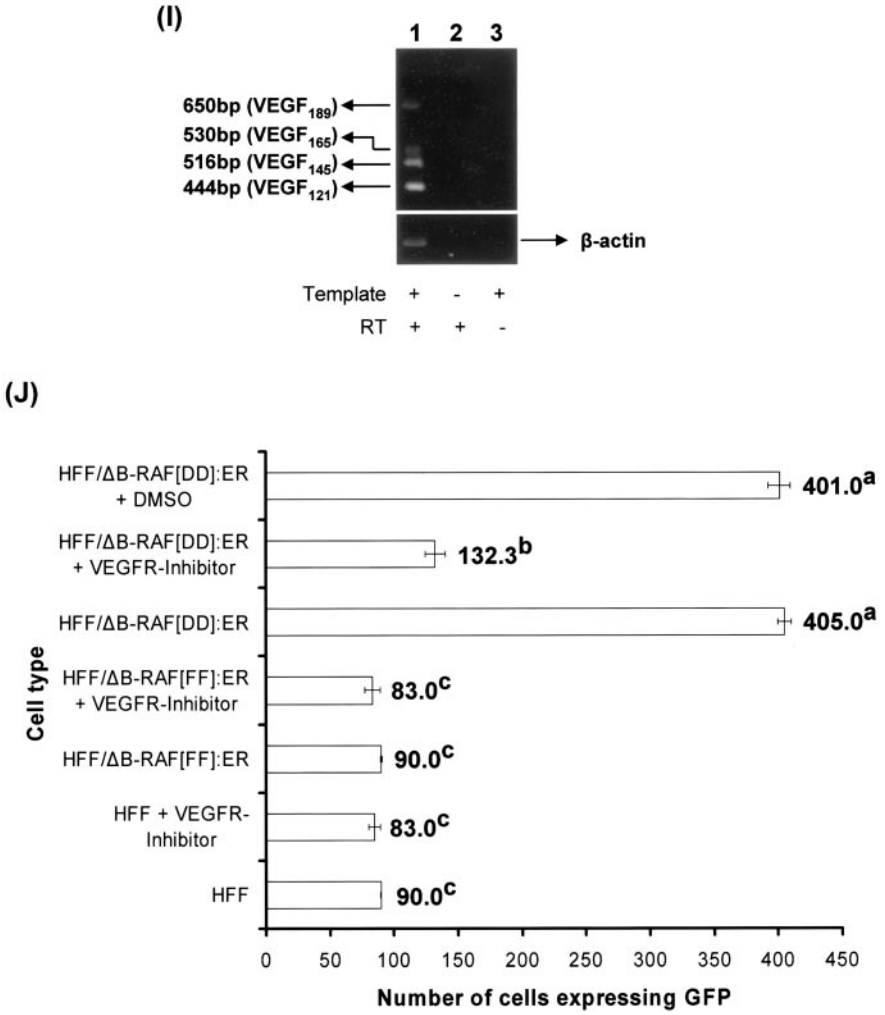


FIG. 1—*Continued.*

Inhibition of VEGF by small interfering RNA (si-RNA)-lowered KSHV infection of HFF/ΔB-Raf$_{[DD]}$:ER cells. To investigate a possible role for VEGF in the enhanced KSHV infection of EST-stimulated HFF/ΔB-Raf$_{[DD]}$:ER cells, we monitored rKSHV.152 infection of target cells that were transfected with si-RNA specific for VEGF as per the protocol recommended by the manufacturer (VEGF siRNA/siAB assay kit; Dharmacon TNA technologies, Lafayette, Colo.). Northern blotting was performed at 0, 12, 24, and 48 h after transfection as per the recommendations of the manufacturer to monitor VEGF mRNA expression (Fig. 2A). The level of VEGF mRNA was significantly suppressed in EST-stimulated HFF/ΔB-Raf$_{[DD]}$:ER cells by si-RNA when compared to nonspecific si-RNA [(NS)si-RNA] control (Fig. 2A). An inhibition of 32 ± 5%, 87 ± 4%, and 75 ± 3% of VEGF mRNA was observed at 12, 24, and 48 h, respectively, after si-RNA transfection in β-estradiol stimulated HFF/ΔB-Raf$_{[DD]}$:ER cells (Fig. 2A). The level of VEGF mRNA was suppressed to undetectable levels in EST-stimulated HFF and HFF/ΔB-Raf$_{[FF]}$:ER cells by 12 h after si-RNA transfection when compared to (NS)si-RNA (Fig. 2A). Based on the above results, we decided to report the data from the EST-stimulated HFF and HFF/ΔB-Raf$_{[DD]}$:ER cells that were transfected with si-RNA as they were more relevant and significant to this study. The VEGF expression in the culture supernatant of si-RNA transfected cells was also monitored by ELISA. We observed a maximal inhibition of VEGF under conditions tested in EST-stimulated HFF/ΔB-Raf$_{[DD]}$:ER cell supernatant by 48 h after si-RNA transfection (Fig. 2B). The level of VEGF was lowered by 85% in EST-stimulated HFF/ΔB-Raf$_{[DD]}$:ER cells (Fig. 2B). We did not observe a significant drop in VEGF expression in EST-stimulated HFF cells that were transfected with si-RNA because the endogenous VEGF expression in untransfected cells is inherently low (Fig. 2B). (NS)si-RNA did not have a significant effect on VEGF expression in target cells (Fig. 2B).

[^{3}H]thymidine-labeled rKSHV.152 bound untransfected and si-RNA-transfected HFF and HFF/ΔB-Raf$_{[DD]}$:ER cells to comparable levels (data not shown). However, we observed a significant drop in rKSHV.152 infection of EST-stimulated HFF/ΔB-Raf$_{[DD]}$:ER cells that were transfected with VEGF-specific si-RNA when compared to either untransfected or cells that were transfected with (NS)si-RNA (Fig. 2C). There was no significant drop in rKSHV.152 infection of EST-stim-

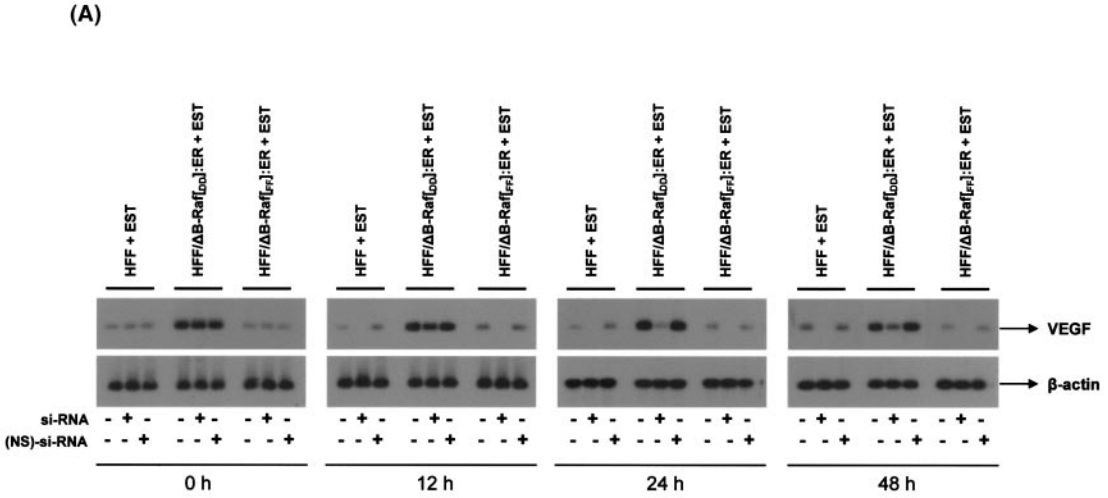


FIG. 2. Inhibition of VEGF expression by si-RNA lowers rKSHV.152 infection of cells. EST-stimulated target cells were untransfected or transfected either with double-stranded si-RNA or (NS)si-RNA controls. (A) At 0, 12, 24, and 48 h posttransfection, total RNA was isolated from the cells and subjected to Northern blotting per standard protocols to monitor VEGF and β-actin mRNA (1). (B) VEGF expression was monitored in cell culture supernatants collected at 0, 12, 24, and 48 h posttransfection by performing ELISA as per protocols mentioned in the legend for Fig. 1H. (C) rKSHV.152 infection of the above cells was performed at 48 h post transfection and analyzed as per standard protocols mentioned in the legend for Fig. 1A. Data presented in panels E and F represent the averages ± SD of three experiments. Average values on the columns with different superscripts are statistically significant ($P < 0.05$) by LSD.

ulated HFF cells that were transfected with VEGF si-RNA (Fig. 2C). It should be noted that silencing the mRNA for VEGF expression in EST-stimulated HFF/ΔB-Raf$_{[DD]}$:ER cells did not completely inhibit rKSHV.152 infection. This could be due to one or both of the following reasons. First, there could also be other factors (other than just VEGF) playing a role in the enhancement of virus entry. Second, the presence of a lag phase between the drop in VEGF mRNA within the cells could have an effect on the VEGF concentrations in the culture supernatant, partly due to the half-life of the already available VEGF. These results indicate that VEGF plays a key role in augmenting KSHV infection of cells at a postattachment stage of entry and that VEGF is not a necessity for KSHV infection of target cells.

Endogenous expression of VEGF$_{121}$ enhances rKSHV.152 infection of cells. We further examined the consequences of expressing endogenous VEGF on KSHV infection by transfecting HFF cells with a mammalian expression vector encoding VEGF$_{121}$. VEGF$_{121}$ was chosen for these experiments, since it is the soluble form of VEGF tested in this study (Fig. 1A) and because it is as functionally active as other isoforms (46).

HFF/V$_{121}$-pcDNA3.1(+) cells produced significantly higher levels of VEGF than HFF/pCDNA3.1(+) and HFF cells (Fig. 3A). VEGF produced by untransfected HFF cells was less than 5 pg/ml. We observed a significant increase in KSHV infection of cells overexpressing VEGF$_{121}$ (Fig. 3B). Interestingly, this increase in rKSHV.152 infection of HFF/V$_{121}$-pcDNA3.1(+) cells was significantly lowered by pretreating cells with anti-human VEGF antibodies for 4 h prior to infection, compared to pretreating cells with preimmune immunoglobulin Gs (IgGs) (Fig. 3B). [^{3}H]thymidine-labeled rKSHV.152 bound HFF, HFF/V$_{121}$-pcDNA3.1(+), and HFF/pcDNA3.1(+) cells to a comparable extent (Fig. 3C). The binding of [^{3}H]thymidine-labeled rKSHV.152 was specifically inhibited by H and not by CSA as was observed in a previous study (2), suggesting that VEGF enhances virus entry at a postattachment stage of infection.

The concentrations of VEGF produced by cells used in this study varied from 0.3 to 92 pg/ml (Fig. 1H). The VEGF concentration monitored in the cell culture supernatants depended upon the volume of the medium and the number of cells per well. With an in vitro tumor model, it was demonstrated that malignant cells secreted approximately 80 to 300 pg/10^6 cells in 24 h (37). In this study, we report an enhanced KSHV infection of cells that endogenously express high levels of VEGF$_{121}$ (Fig. 3). However, it took ≥500 ng of supplemented soluble VEGF/ml to enhance KSHV infection. This could be due to at least two reasons. First, there are at least five different isoforms of VEGF (21, 45). Cells express all of these forms of VEGF simultaneously. However, VEGF$_{121}$, which lacks the heparin binding motif, diffuses better than VEGF$_{165}$ and VEGF$_{189}$, because it does not bind to heparan sulfate (HS) expressed on the cell surface (46). Hence, the VEGF$_{121}$ isoform is more readily detected by in vitro assays used to monitor serum and plasma levels, compared to the other isoforms. Second, under natural conditions, the cells are primed over a long period of time with all of the different isoforms of VEGF, compared to treatment of cells with soluble VEGF for only 1 h (Fig. 1A).

VEGF and its receptors have been proposed to play major roles in KS pathogenesis (4, 29). VEGF is thought to be 50,000 times more potent than histamine on a molar basis at increasing the permeability of microvessels to plasma macromolecules (55). In addition, it plays a central role in promoting hyperpermeability of tumor vessels, as well as tumor neovascularization (14, 47). All of these unique characteristics have made both VEGF and VEGFR targets for the treatment of KS and

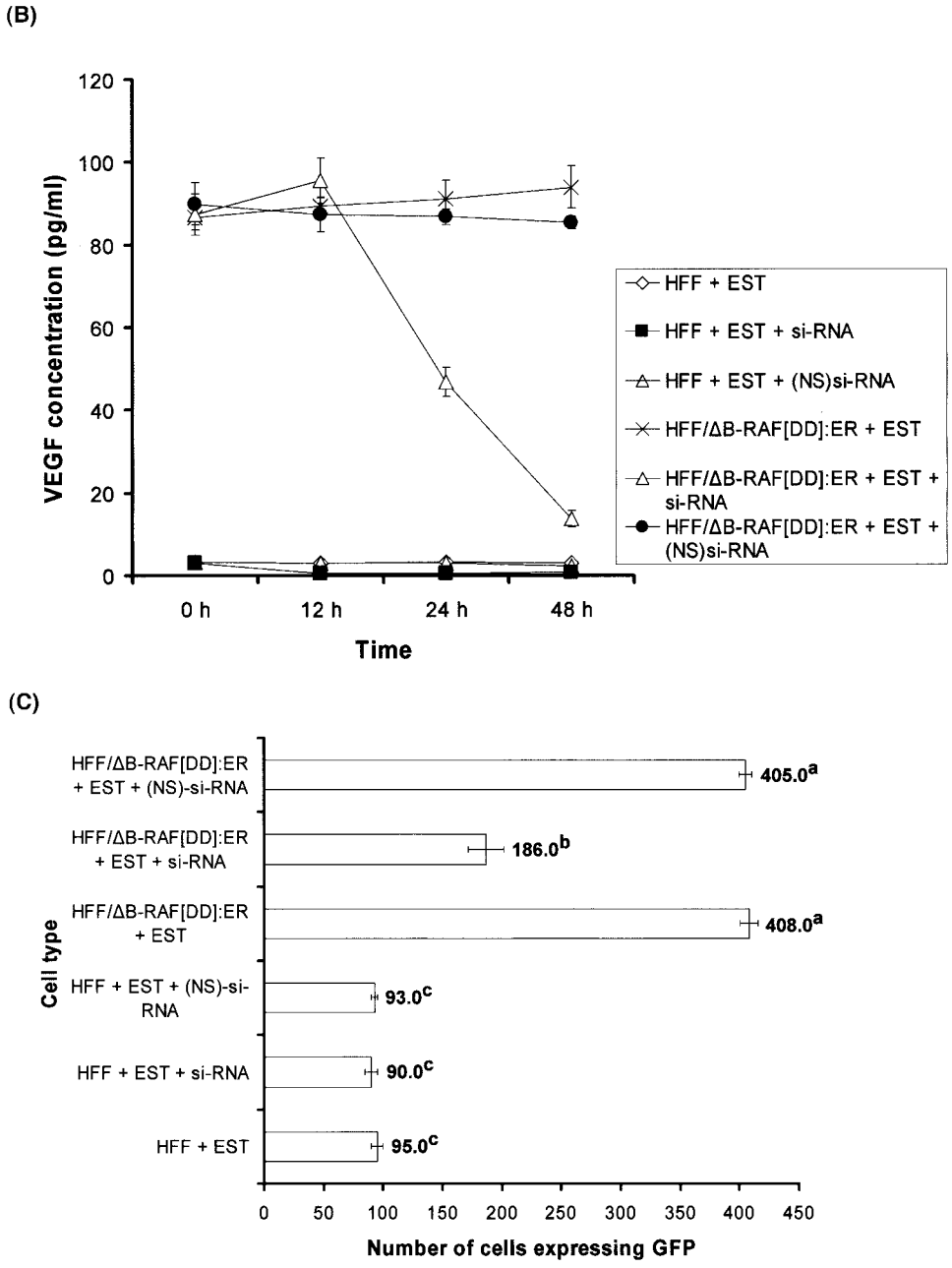
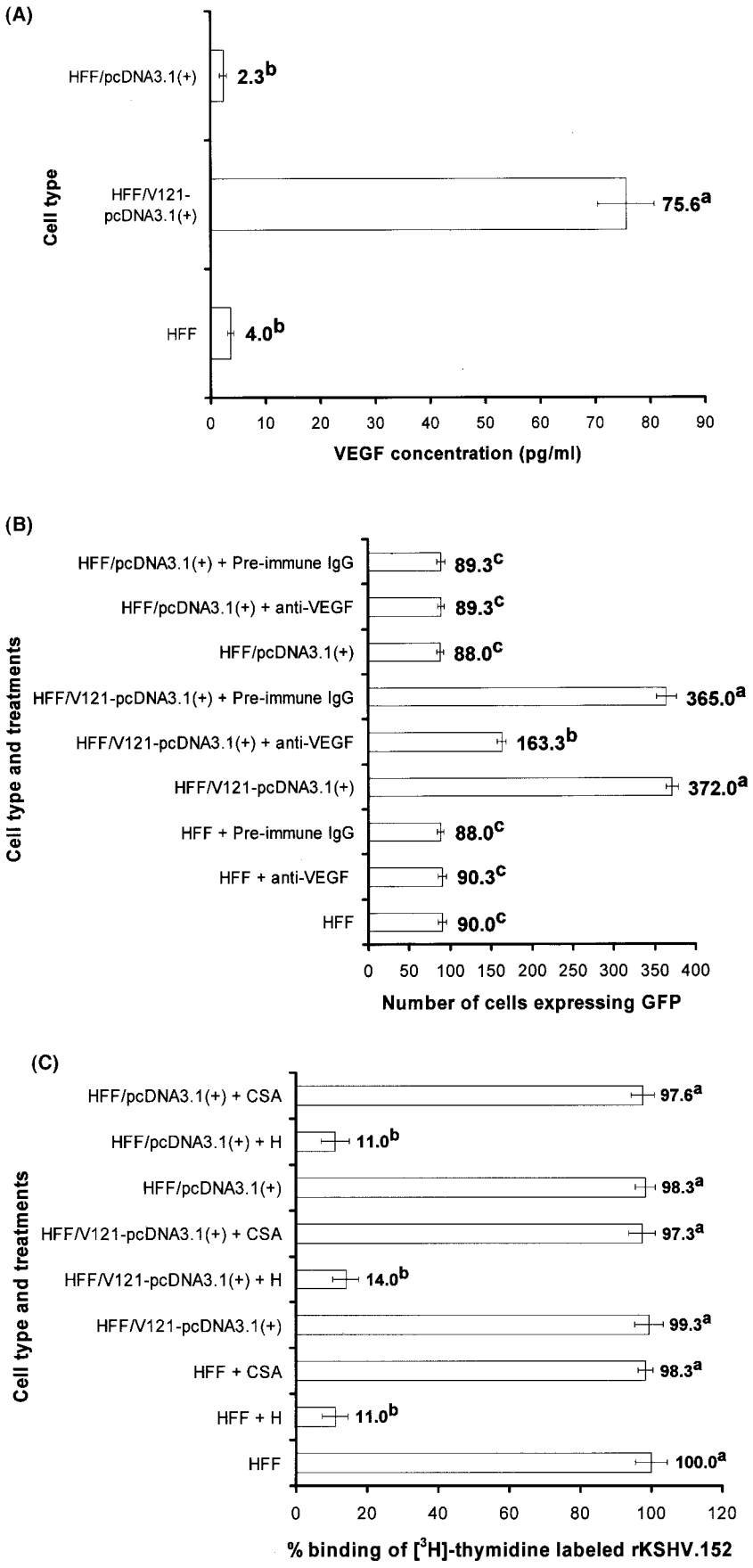


FIG. 2—*Continued.*

other tumor conditions (42, 52). In this study, we used HFF cells that express only VEGFR-1; expression of VEGFR-2 was undetectable by RT-PCR (1). VEGFR-1 (also known as the fms-like tyrosine kinase [Flt]-1) is expressed on the cell surface as a 180- to 185-kDa homodimeric glycoprotein with seven Ig-like extracellular regions (17). VEGFR-1 is expressed primarily on endothelial cells, but studies continue to demonstrate new cell types that express this receptor (33). VEGFR-1 has high affinity (10-fold higher than VEGFR-2) for VEGF and placental growth factor (7, 16), but compared to VEGFR-2, the tyrosine kinase activity of VEGFR-1 is substantially weakened (by about 1/10), which makes autophosphorylation difficult to detect (51). It is for this reason that the mechanism of signaling utilized by VEGFR-1 has not been well defined (13). Binding of ligand initiates receptor dimerization and autophosphorylation, a prerequisite for signal transduction (15). It has been demonstrated that VEGFR-1 has the ability to induce phosphorylation of gamma phospholipase C in vitro as well as coupling with signal transduction molecules such as extracellular signal-regulated kinases 1 and 2, Crk, and SHP-2 upon binding VEGF or placental growth factor (33). VEGFR-1-mediated transduction of cellular signaling produces an assortment of cellular responses, many of which differ between various cell types. Some significant effects of VEGFR-1 signaling


(A)
HFF/pcDNA3.1(+)
2.3b
HFF/V121-pcDNA3.1(+)
75.6a
HFF
4.0b
Cell type
0 10 20 30 40 50 60 70 80 90
VEGF concentration (pg/ml)
(B)
HFF/pcDNA3.1(+) + Pre-immune IgG
89.3c
HFF/pcDNA3.1(+) + anti-VEGF
89.3c
HFF/pcDNA3.1(+)
88.0c
HFF/V121-pcDNA3.1(+) + Pre-immune IgG
365.0a
HFF/V121-pcDNA3.1(+) + anti-VEGF
163.3b
HFF/V121-pcDNA3.1(+)
372.0a
HFF + Pre-immune IgG
88.0c
HFF + anti-VEGF
90.3c
HFF
90.0c
Cell type and treatments
0 50 100 150 200 250 300 350 400
Number of cells expressing GFP
(C)
HFF/pcDNA3.1(+) + CSA
97.6a
HFF/pcDNA3.1(+) + H
11.0b
HFF/pcDNA3.1(+)
98.3a
HFF/V121-pcDNA3.1(+) + CSA
97.3a
HFF/V121-pcDNA3.1(+) + H
14.0b
HFF/V121-pcDNA3.1(+)
99.3a
HFF + CSA
98.3a
HFF + H
11.0b
HFF
100.0a
Cell type and treatments
0 20 40 60 80 100 120
% binding of [3H]-thymidine labeled rKSHV.152

include recruitment of monocytes and macrophages to sites of angiogenesis (34), negative modulation of endothelial cell division in embryogenesis (23, 36), hematopoietic repopulation in adult mice (26), VEGF-dependent actin reorganization and migration (35), regulation of sprout formation and migration in endothelial cell morphogenesis (36), cross talk and/or transphosphorylation of VEGFR-2 (5), and both positive and negative roles in angiogenesis due to production of membrane-bound and soluble forms (24, 33); VEGFR-1 has also been linked to the pathogenesis of several tumors, especially leukemia (25). Contrarily, VEGFR-1 may act as a decoy receptor, with the task of requisitioning extracellular VEGF on the cell surface to increase interactions with VEGFR-2 (51). At this point, our knowledge of the influence of VEGFR-2 on KSHV entry is limited. However, based on the fact that VEGF mediates its effect via both VEGFR-1 and VEGFR-2, which have overlapping functions (6, 40), we hypothesize that VEGFRs play a role in KSHV entry in addition to mediating pathogenesis. This could occur at two levels. First, VEGF-VEGFR signaling can initiate a Raf-associated activation by extracellular signal-regulated kinases 1 and 2, which have been shown in a previous study to enhance the spread of KSHV and thus mediate pathogenesis (1). Second, actin reorganization mediated by VEGF-VEGFR signaling may play a vital role in virus entry by endocytosis (18, 28).

Our results demonstrate that VEGF is not actually a requirement for KSHV infection of target cells. HFF cells that inherently express low levels of Raf and VEGF support KSHV infection (1); however, both Raf (1) and VEGF serve to enhance the already existing level of KSHV infection as observed in HFF/ΔB-Raf$_{[DD]}$:ER cells (Fig. 1A). Taking the results together, we propose that either overexpression or oncogenic mutations in the Raf gene may lead to enhanced VEGF expression, resulting in KSHV spread and dissemination, which is the key factor in pathogenesis. Such a Raf-induced VEGF expression leading to tumor formation has been documented previously (41, 48). Our present studies are focused on deciphering the correlation between Raf-VEGF expression in KS and other KSHV-associated pathogenesis.

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FIG. 3. Human VEGF$_{121}$ enhances rKSHV.152 entry in HFF cells. (A) The expression of VEGF by target cells was analyzed by ELISA as per protocols described in the legend for Fig. 1H. (B) Effect of endogenous VEGF on rKSHV.152 infection of HFF cells was analyzed. The full-length VEGF$_{121}$ gene (11) was subcloned from pBluescript SK(minus) (Stratagene, La Jolla, Calif.) into the BamHI/EcoRI sites of pcDNA3.1(+) (Invitrogen, Carlsbad, Calif.), a eukaryotic expression vector containing the HCMV immediate-early promoter to create the VEGF$_{121}$/pcDNA3.1(+) clone. HFF cells were transfected with either pcDNA3.1(+) or VEGF$_{121}$/pcDNA3.1(+) with Lipofectamine 2000 (Invitrogen) as per the manufacturer's recommendations. Stably transfected cells were isolated by incubating cells in DMEM containing 500 μg of G418/ml as per previous protocols (1). The cells were referred to as HFF/pcDNA3.1(+) and HFF/VEGF$_{121}$-pcDNA3.1 cells, respectively. These cells were treated with DMEM alone or DMEM containing either preimmune IgGs or anti-VEGF antibodies for 4 h at 37°C. These cells were infected with rKSHV.152, and the extent of infection was monitored as per protocols in the legend for Fig. 1A. (C) VEGF enhances rKSHV.152 infection at a post-cell-attachment stage of entry. The ability of purified [^{3}H]thymidine labeled rKSHV.152 (2,830 cpm) to bind HFF, HFF/V$_{121}$-pcDNA3.1(+), and HFF/pcDNA3.1(+) cells was analyzed as per the protocols outlined in the legend for Fig. 1G. Data represent the average $\pm$ SD of three experiments. Average values on the columns with different superscripts are statistically significant ($P < 0.05$) by LSD.

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